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Prokaryotic expression of the *Mycobacterium tuberculosis PhoY2* gene and its diagnostic utility in various populations infected with tuberculosis

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ABSTRACT: Early and timely diagnosis is crucial for reducing the transmission of tuberculosis. In this study, we constructed and expressed PhoY2, a persistent infection related antigen of *Mycobacterium tuberculosis* and evaluated its potential as a diagnostic target through population experiments. The immunogenicity of the recombinant PhoY2 (rPhoY2) protein in *M. tuberculosis* infected individuals was evaluated using whole blood interferon-gamma (IFN-γ) release assay (WBIA) and serum specific antibody detection. Further, the diagnostic value, sensitivity, and specificity of rPhoY2-WBIA and anti-rPhoY2 specific antibody in different *M. tuberculosis* infected populations were analyzed using receiver operating characteristic. The results indicated that rPhoY2 can stimulate specific IFN-γ production in active tuberculosis (ATB) and latent tuberculosis infection (LTBI), levels of which were significantly higher than that in healthy controls (HCs). The anti-rPhoY2 specific IgG levels of LTBI were significantly higher than those of ATB and HCs. The cutoff value of rPhoY2-WBIA for diagnosing *M. tuberculosis* infection is 309.2 pg/ml with a sensitivity of 72.2% and a specificity of 93.7%. The cutoff value of anti-rPhoY2 specific IgG for diagnosing LTBI is 0.1765 with 61.5% sensitivity and 75% specificity. The agreement rate between rPhoY2-WBIA combined with specific antibodies and clinical diagnosis for the three populations is 69.9%. In summary, the combination of rPhoY2-WBIA and specific antibody detection has certain value in distinguishing different populations infected with *M. tuberculosis*, especially in excluding the infection with high specificity. However, its sensitivity should be further enhanced for differentiating ATB and LTBI.

KEYWORDS: *Mycobacterium tuberculosis*, PhoY2, prokaryotic expression, immunogenicity

INTRODUCTION

Tuberculosis (TB) continues to pose a significant global public health challenge. As stated by the World Health Organization (WHO), the year 2021 witnessed approximately 10.6 million newly reported TB cases across the globe, leading to 1.6 million deaths attributed to this infectious disease [1]. Moreover, around 25% of the global population is infected with *Mycobacterium tuberculosis*, and within 2 years, 5% of those infected can progress to active tuberculosis (ATB), while the host immune response controls and triggers latent tuberculosis infection (LTBI) in the remaining 90–95% of cases. Nevertheless, 5–10% of individuals carrying latent infections may develop ATB over their lifetime as a result of endogenous infections [2]. Timely diagnosis, prompt treatment, and effective prevention are essential in controlling TB transmission. Therefore, it is of paramount importance to develop rapid, sensitive, and cost-effective diagnostic methods for TB as well as new vaccines for protection. Unfortunately, the current diagnosis and vaccine development for TB are far from ideal. Existing clinical diagnostic methods for TB (such as traditional microbiological tests, molecular biology tests, etc.) have certain limitations, particularly in the detection of latent infection carriers [3,4]. Immunological testing methods can partially diagnose latent infection, but the current commercialized tests, including various antibody tests and tuberculin skin test (TST), suffer from inadequate sensitivity and specificity, leading to reduced diagnostic accuracy for TB. Furthermore, the Bacillus Calmette-Guérin (BCG) vaccine, the sole widely employed vaccine for TB, is highly effective in preventing severe TB manifestations in children but lacks effectiveness in preventing LTBI and has limited protective effects in adults [5,6]. Hence, TB vaccine researchers should continue to pursue the screening of high-quality *M. tuberculosis* protein antigens, the development of immunological diagnostic methods with improved accuracy, and the creation of novel and more potent vaccines.

Studies have discovered that the *PhoY2* gene (*Rv0821c*) in *M. tuberculosis* exhibits homology with the *PhoU* gene in *Escherichia coli* (*E. coli*) and plays a role in the persistent infection of *M. tuberculosis* in the human body [7,8]. The current limited research on *PhoY2*, primarily concentrated on its pathogenic mechanisms, has not yet determined its clinical significance in prevention and diagnosis. The aim of the present study is to engineer and produce recombinant PhoY2 (rPhoY2) protein and assess its value for diagnosing TB infection in different populations.

MATERIALS AND METHODS

Materials

Strains and plasmids

M. tuberculosis H37Rv standard strain was obtained from the Tuberculosis Branch of the Shanxi Provincial

Institute for Disease Control and Prevention (Shanxi, China). *E. coli* DH5α strain and BL21(DE3) were purchased from Sangon Company (Shanghai, China). Plasmid pET30b(+) was obtained from our laboratory (Shanxi, China).

Reagents

Restriction endonucleases, *Nde*I and *Xho*I, and medium molecular weight protein marker were purchased from GE Company (Shanghai, China). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Sigma Company (St. Louis, MO, USA). DNA Marker D2000 was purchased from Dongsheng Reagent Company (Guangzhou, China). Bacterial Genomic DNA Extraction Kit, mouse anti-His polyclonal antibody, and soluble single-component TMB substrate solution were purchased from TIANGEN Company (Beijing, China). Plasmid Miniprep Kit was purchased from Axygen Company (Tewksbury, MA, USA). Ni-NTA Column was purchased from Invitrogen Company (Carlsbad, CA, USA). Enzyme-linked immunosorbent assay (ELISA) polystyrene plate was purchased from Jet Biofil Company (Guangzhou, China). HRP Goat-anti-Human IgG was purchased from Proteintech Company (Rocky Hill, NJ, USA). AxyPrep DNA Gel Extraction Kit and AxyPrep PCR Clean-up Kit were purchased from Axygen Company. Human interferon-gamma (IFN-γ) ELISA Kit was purchased from MultiSciences Company (Hangzhou, China). Bicinchoninic Acid (BCA) protein analysis kit was purchased from Beyotime Company (Shanghai, China). *PhoY2* upstream and downstream primers and oligodeoxynucleotide sequence (CpG ODN) were synthesized by Synbio Biotechnology Company (Suzhou, China).

Plasma samples

A total of 28 ATB and 26 LTBI were randomly selected from the Department of Infection, Heping Hospital, Changzhi Medical College. The diagnosis of ATB and LTBI was based on the diagnostic criteria for TB issued by the National Health Commission of the People's Republic of China in 2008. The final diagnosis references included sputum smear, TB culture results, chest X-ray, TST, and clinical symptoms. Forty-nine individuals from the Health Examination Center were selected as healthy controls (HCs). HCs had no history of close contact with TB patients and had negative TST results. The acquisition of human serum was approved by the Ethics Committee of Heping Hospital, Changzhi Medical College (Ethics Approval No: (2022)004). All subjects provided informed consent. Fresh anticoagulated whole blood samples were collected in sterile conditions at a volume of 3 ml per person. Within 6 h, 0.5 ml per well of the fresh anticoagulated whole blood was added to sterile 24-well plates. To each well, 20 μl of antigen was added, 20 μl of phosphate buffer saline (PBS) was added as a negative control, and 20 μl of phytohemagglutinin (PHA) was added as a positive control. The mixture was gently shaken to ensure proper mixing. The plates were then incubated at 37 °C for 20–24 h. Plasma was collected in sterilized EP tubes and immediately frozen at −20 °C for further analysis.

Methods

Construction of PhoY2 recombinant prokaryotic expression plasmid

The gene sequence of *PhoY2* was retrieved from Genbank. Using DNAMAN software and the pET30b(+) vector map, the optimal multiple cloning restriction enzyme cutting sites *Nde*I and *Xho*I were determined. Specific upstream and downstream primers were designed using Primer premier 5.0. The sequences of the primers used in the experiment were 5′ TTCCATATGCGGACCGCCTACCATGAGCA3′ for the upstream primer P1 and 5′ATCTCGAGGGGAAAGGC GCCGGTCG3′ for the downstream primer P2. The *PhoY2* gene was then amplified from the H37Rv genome using PCR and cloned into the *E. coli* expression vector pET30b(+) using molecular cloning techniques. Plasmids were extracted from kanamycin-resistant colonies and identified by double enzyme digestion with textitNdeI and *Xho*I. The correctness of the inserted target gene was confirmed by sequencing. The identified recombinant plasmid was subsequently transformed into *E. coli* DH5α for large-scale cultivation to obtain the recombinant plasmid.

Recombinant protein expression of rPhoY2

The pET30b(+)-PhoY2 plasmid was introduced into *E. coli* BL21(DE3) competent cells containing $CaCl_2$ through the heat shock method. Protein expression was induced by adding IPTG inducer at a final concentration of 0.5 mmol/l. The cells were then sonicated, and the protein was purified using Ni-NTA affinity chromatography. The purity of the purified protein was determined using 15% SDS-PAGE. For western blot analysis, mouse-derived His monoclonal antibody was used as the primary antibody, and HRP-labeled goat anti-mouse IgG was used as the secondary antibody. Successful identification of the protein was achieved using this method. Next, the purified protein was subjected to gradient dialysis at 4 °C as per the instructions provided by the Ni-NTA column purification system. The effectiveness of the purification process was verified through 12.5% SDS-PAGE electrophoresis. To remove endotoxins, an endotoxin removal kit was utilized, resulting in a final endotoxin level below 0.1 EU/ml. Finally, the protein concentration was quantified using a BCA assay kit.

Whole blood IFN-γ release assay (WBIA)

A peripheral blood sample of 0.5 ml was collected from each subject using a heparin anticoagulant tube. The collected samples were then exposed to 5 μg/well of rPhoY2 protein and incubated at 37 °C for a period of 18–24 h. PHA and PBS were used as the positive and negative controls, respectively. The levels of IFN-γ release in each well were quantified according to the guidelines provided by the human IFN-γ ELISA kit. It is crucial to take into account the background value observed in the PBS negative control group when calculating the final IFN-γ values.

ELISA detection of specific IgG levels against PhoY2 protein in different population sera

The rPhoY2 protein was diluted to a concentration of 5 μg/ml using 0.05 M pH 9.6 carbonate coating buffer. Following this, 100 μl of the diluted protein was added to each well of an ELISA polystyrene plate and allowed to incubate at 4 °C overnight. The plate was then washed 3 times using 0.05% PBST and subsequently blocked with 1% bovine serum albumin. The serum samples were diluted 1:200 and incubated at 37 °C for 1 h with 100 μl per well. Subsequently, HRP-conjugated goat anti-human IgG, diluted 1:5000, was added to each well and incubated at 37 °C for 1 h. Finally, 100 μl of TMB substrate was added to each well, and the color development was allowed to proceed for 15 min. The reaction was halted by adding 100 μl of 1 M H_2SO_4. The optical density (OD) values for each well were then determined at 450 nm using an ELISA reader.

Statistical analysis

Data analysis was conducted using SPSS 22.0 software. To compare the specific levels of IFN-γ and IgG between different populations, a non-parametric Kruskal-Wallis one-way analysis was employed and if there were significant differences among multiple groups, post-hoc pairwise comparisons were conducted to assess the specific group differences. The χ^2 test was employed to compare the disparity in sex distribution across various populations. The receiver operating characteristic (ROC) curve area and curve coordinates were obtained using SPSS 22.0, including the sensitivity and 1-specificity for each coordinate point. The specificity for each coordinate point was obtained by 1-(1-specificity). The coordinate point corresponding to the maximum sum of sensitivity and specificity was the cutoff value. The number of individuals whose clinical diagnosis matched with rPhoY2-WBIA combined with specific antibodies (i.e., the total number diagnosed as ATB, LTBI, and HCs simultaneously by both methods) divided by the total number of individuals in the study population gives the diagnostic concordance rate. The significance level α was set at 0.05 to determine statistical significance.

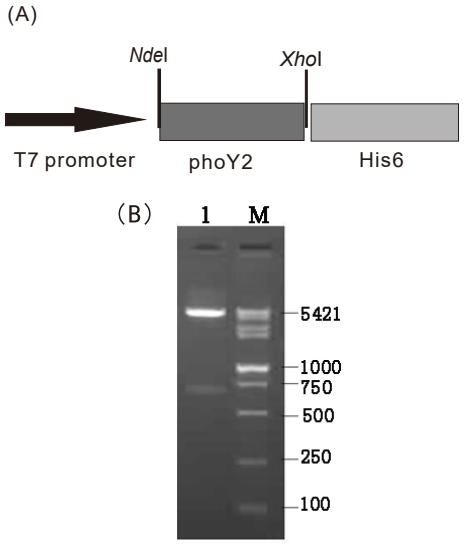

Fig. 1 Construction and identification of recombinant plasmid pET(+)-PhoY2. (A) Schematic diagram illustrating the construction of the recombinant plasmid pET(+)-PhoY2. (B) Enzymatic digestion analysis of the recombinant plasmid pET(+)-PhoY2. Lane 1, DNA fragments of target gene and vector; lane M, DNA marker.

RESULTS

Construction and identification of recombinant plasmid

The recombinant plasmid pET(+)-PhoY2 was verified through *Nde*I and *XhoI* double digestion assay (Fig. 1A). The subsequent analysis using 1% agarose gel electrophoresis exhibited the presence of 2 distinct DNA fragments. The fragment of approximately 690 bp corresponded to the target gene, while the fragment of 5,421 bp represented the vector (Fig. 1B). The observed fragment sizes were consistent with the theoretical design.

Expression, purification, and identification of rPhoY2

The recombinant plasmid pET(+)-PhoY2 was introduced into *E. coli* BL21(DE3) for heterologous protein expression. Following induction with IPTG for a duration of 4 h, there was a significant increase observed in the expression level of rPhoY2. Solubility analysis validated the presence of the recombinant protein within the form of inclusion bodies. The purification of rPhoY2 protein was carried out utilizing the Ni-NTA affinity chromatography system under denaturing conditions, as confirmed by SDS-PAGE (Fig. 2A) and western blot analysis (Fig. 2B). The determined molecular weight of the expressed protein was approximately 23.5 kDa.

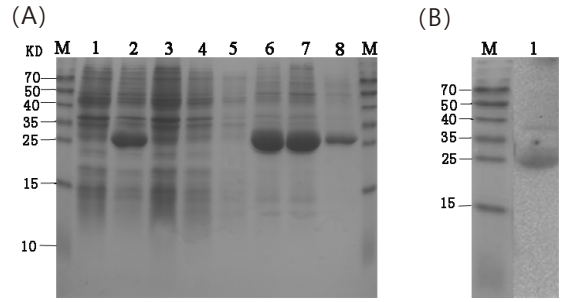

Fig. 2 Expression, purification, and identification of the rPhoY2 protein. (A) Expression and purification of the rPhoY2 protein. Lane M, protein marker (kDa); lane 1, recombinant bacteria without induction control; lane 2, induced recombinant bacteria whole cell lysate with IPTG; lane 3, supernatant after ultrasonic disruption of induced recombinant bacteria with IPTG; lane 4, protein eluate from nickel column chromatography; lane 5, eluate after washing with 8 mol/l urea binding buffer; and lane 6–8, protein elution from nickel column chromatography. (B) Western blot analysis of the rPhoY2 protein. Lane M, protein marker (kDa); lane 1, purified rPhoY2.

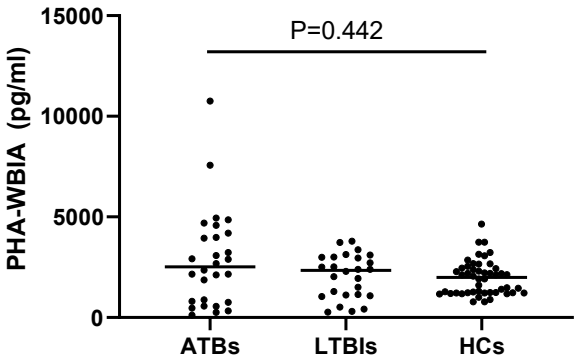

Fig. 3 PHA stimulating the IFN-γ response level of different populations.

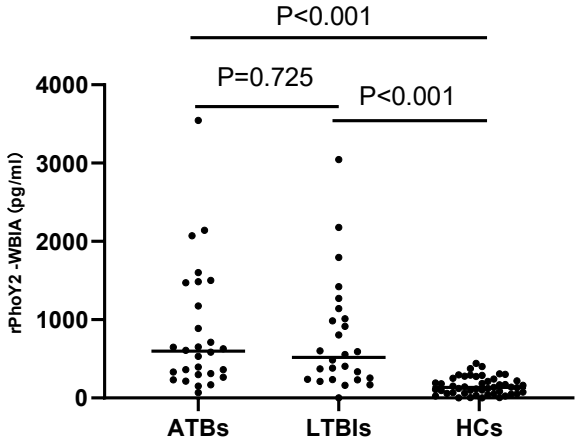

Fig. 4 The response levels of rPhoY2 specific IFN-γ in different populations.

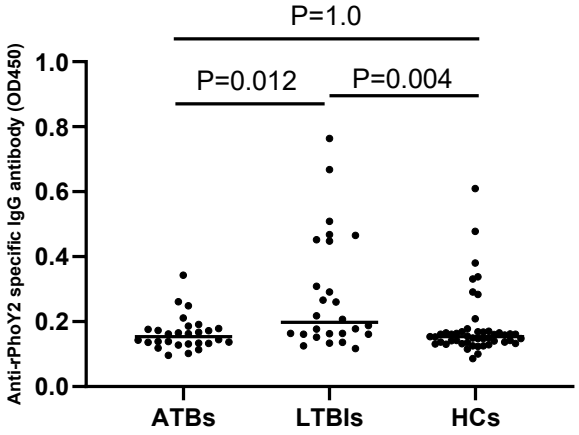

Fig. 5 The response levels of anti-rPhoY2 specific IgG in different populations.

Levels of rPhoY2-specific IFN-γ and anti-rPhoY2-specific IgG responses in different population groups

The median age of the ATB, LTBI, and HC groups were 28, 26, and 27 years, respectively. There was no significant difference in age between different populations ($p = 0.91$). The number of males and females in different populations is shown in Table 1. After χ^2 testing, there was no significant difference between males and females in different populations ($p = 0.53$). After PHA stimulation, there was no significant difference in the levels of IFN-γ in peripheral blood between ATB, LTBI, and HCs ($p = 0.44$), indicating that there was no significant difference in immunity between different populations (Fig. 3). Significant disparities were observed in the concentrations of particular IFN-γ and anti-rPhoY2-specific IgG responses generated subsequent to rPhoY2 stimulation in the peripheral blood of individuals with ATB, LTBI, and HCs. Further pairwise comparisons revealed that the levels of specific IFN-γ triggered by rPhoY2 stimulation were markedly elevated in ATB and LTBI when compared to HCs. However, no statistically significant difference was observed in the levels of specific IFN-γ between ATB and LTBI (Fig. 4). On the other hand, the levels

Table 1 The number of male and female in different groups.

Grouping gender	Male	Female	Total
ATB	14	14	28
LTBI	14	12	26
HC	30	19	49
Total	58	45	103

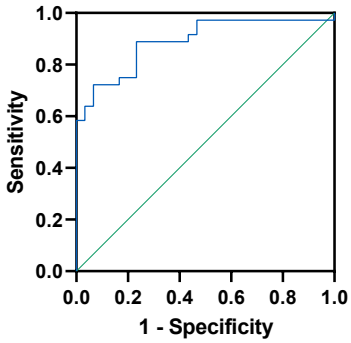

Fig. 6 ROC of rPhoY2-WBIA diagnosis of *M. tuberculosis* infection.

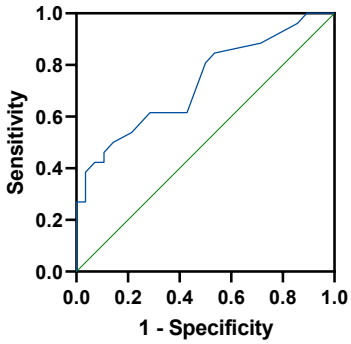

Fig. 7 ROC of anti-rPhoY2 specific IgG in diagnosing LTBI.

of specific anti-rPhoY2 IgG differed significantly with noticeably higher levels observed in LTBI as compared to ATB and HCs, while no statistically significant difference was found between ATB and HCs (Fig. 5).

rPhoY2-WBIA joint specific antibody diagnostic for TB infection in different populations

The diagnostic value of rPhoY2-WBIA in detecting *M. tuberculosis* infection was determined through the ROC curve analysis, yielding an area under the curve of 0.891 (95% CI: 0.829–0.953, $p < 0.001$) with a cutoff value of 309.2 pg/ml. The sensitivity and specificity were determined to be 72.2% and 93.7%, respectively (Fig. 6). Additionally, the diagnostic value of rPhoY2-specific IgG for diagnosing LTBI was further analyzed, resulting in a cutoff value of 0.1765 and an area under the curve of 0.727 (95% CI: 0.592–0.863, $p = 0.004$). The sensitivity and specificity were determined to be 61.5% and 75%, respectively (Fig. 7). The agreement rate between the rPhoY2-WBIA combined specific antibody diagnosis and clinical diagnosis across the 3 populations was 69.9% (Table 2).

Table 2 Consistency rate of rPhoY2-WBIA combined with specific antibodies in diagnosing 3 populations and clinical diagnosis.

Joint diagnosis clinical diagnosis	ATB	LTBI	HC	Total
ATB	16	8	1	25
LTBI	5	10	2	17
HC	7	8	46	61
Total	28	26	49	103

DISCUSSION

Immunological testing is a crucial approach for aiding the diagnosis of TB in clinical practice, particularly for the identification of LTBI. The primary techniques utilized in immunological examination encompass IFN-γ related detection and antibody detection. IFN-γ related detection is a widely employed approach for identifying target antigens for TB vaccines, and it also offers diagnostic value in TB [9,10]. IFN-γ can be stimulated by *M. tuberculosis* specific antigens (such as ESAT-6 and CFP-10), which only exist in *M. tuberculosis* and not in BCG. Indirectly assessing the presence of *M. tuberculosis* infection or latent infection can be achieved by measuring the level of IFN-γ produced by the patient's immune system. During the screening of target antigens for TB vaccines, elevated levels of IFN-γ signify the potential of vaccine candidate strains to elicit robust immune responses in the human body. Therefore, the level of IFN-γ can serve as a pivotal indicator for evaluating the immune protection conferred by vaccines, and its expression across different populations aids in the auxiliary diagnosis of TB. *M. tuberculosis* does not hinder the normal development of plasma cells, and antibodies play a protective role in *M. tuberculosis* infection by activating complement, enhancing cellular immunity, and modulating inflammatory responses, among others [11,12]. While the diagnostic significance of specific antibody detection in TB is not substantial, the response of TB patients to antigens varies among individuals. However, certain specific antibodies have been proposed as biomarkers for ATB, and numerous *M. tuberculosis* specific antibodies have demonstrated potential diagnostic value [13,14,15].

Based on the research findings, it is evident that the expression level of specific IFN-γ induced by rPhoY2 is elevated in both TB-infected and healthy populations. This suggests that the gene holds promise as a target antigen for TB vaccines. Moving forward, we will continue our investigation into the efficacy of this gene as a target antigen for TB prevention and control. Additionally, this study assessed the diagnostic value of the rPhoY2 protein for different populations infected with *M. tuberculosis*. The results demonstrated that rPhoY2-WBIA can effectively discern between TB-infected and non-infected populations but cannot differentiate between ATB and LTBI. The sensitivity and specificity of *M. tuberculosis* infection diagnosis using rPhoY2-WBIA are comparable

to that of IGRAs, which have a sensitivity of 78% to 100% and a specificity of 87.5% to 100% in clinical TB detection [16,17]. Furthermore, rPhoY2-specific antibody detection enables the diagnosis of LTBI in the infected population. However, its sensitivity for diagnosing LTBI is only 61.5%, potentially influenced by varying stages of latent infections or the reconstitution effect of the recombinant protein and exposure to its antigenic epitopes. In subsequent experiments, we will employ more advanced techniques to generate this antigen and obtain a deeper understanding of its immunological properties.

The rPhoY2-WBIA and specific antibody combination developed in this study holds significant application value for diagnosing *M. tuberculosis* infection; however, further enhancements are required for diagnosing LTBI. Furthermore, considering the limited sample size in this study, there might be slight deviations in the test results. Therefore, additional samples are necessary to further validate the effectiveness of rPhoY2-WBIA in combination with specific antibody diagnosis across diverse populations with TB infection.

One significant factor contributing to the ineffectiveness of BCG in preventing TB is the ability of *M. tuberculosis* to establish latent infection, during which the bacteria exist in a dormant state with minimal or no replication [5,6]. Research has demonstrated that the gene expression of *M. tuberculosis* is distinct under stress conditions compared to the normal proliferative state under favorable conditions. Additionally, various stress conditions (such as nutrient deficiency, low oxygen, low pH, or high NO) induce distinct gene expression profiles [18,19,20]. Novel TB vaccines under domestic and international research focus on *M. tuberculosis* secreted antigens during the latent phase. However, these antigens are downregulated to different extents during latent *M. tuberculosis* infection, thus impeding the protective effectiveness of these vaccines against latent TB. Consequently, some researchers have integrated antigens expressed during the latent phase of *M. tuberculosis* infection into the development of innovative vaccines as a means to address the shortcomings of current vaccines in preventing latent TB [21,22]. This study successfully generated the rPhoY2 protein, which was specifically recognized by peripheral blood T cells in individuals infected with *M. tuberculosis*, as confirmed by serological experiments. This target antigen shows promise for further investigation and could be incorporated in the development of fusion protein vaccines for TB.

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